Effect of Decellularized Cartilage

by Ferdiansyah Ferdiansyah

Submission date: 29-Jan-2019 10:33AM (UTC+0800)
Submission ID: 1069871275
File name: Dr_FER-DNU-TRI_JBBBE_vol_35_2018.pdf (1.29M)
Word count: 3924
Character count: 23372

Journal of Biomimetics, Biomaterials and Biomedical Engineering
ISSN: 2296-9845, Vol. 35, pp 67-76
doi: 10.4028/www.scientific.net/JBBBE.35.67

Submitted: 2017-09-08
Revised: 2017-11-01
Accepted: 2017-11-02
Online: 2018-01-19

Effect of Decellularized Cartilage Bovine Scaffold and Hypoxic Condition on Stem Cell Differentiation to Chondrocyte: An *In Vitro* Study

Ferdiansyah Mahyudin[1,a,*], Dwikora Novembri Utomo[1,b], Tri Wahyu Martanto[1,c], Aries Rakhmat Hidayat[2,d], Linta Meyla Putri[3,e]

[1]Department of Orthopedic and Traumatology, Faculty of Medicine, Universitas Airlangga/Dr. Soetomo General Hospital, Indonesia

[2]Resident in Department of Orthopedic and Traumatology, Faculty of Medicine, Universitas Airlangga/Dr. Soetomo General Hospital, Indonesia

[3]Magister Student in Faculty of Public Health, Universitas Airlangga, Indonesia

[a]ferdyortho@gmail.com, [b]dwikora_utomo@yahoo.com, [c]orthotri2000@gmail.com, [d]ariesrakhmat@gmail.com, [e]lintameyla@gmail.com

*Corresponding author: ferdyortho@gmail.com

Keywords: cartilage defect, decellularized cartilage bovine scaffold, hypoxic stem cell

Abstract. Autologous Chondrocyte Implantation (ACI) has been established for years to treat cartilage defect. Application of tissue engineering has advantages over ACI as tissue engineering requires simpler procedures without leaving morbidity at the donor site. Decellularized bovine cartilage scaffold and hypoxic stem cell differentiation were used in this in vitro experimental study. Comparative test was done between three study groups using bone marrow mesenchymal stem cells treated in three different conditions: growth factor-rich chondrogenic medium, scaffold without growth factor, and combination of both. Each group was given two oxygen tension conditions of normoxia and hypoxic within phase of stem cell differentiation. Immunohistochemical examinations on SOX9, RUNX2, and collagen type II were done for evaluation. After 5-week treatment, the result showed that the highest expression SOX9 and collagen type II were found within the group that used the combination of both scaffold and chondrogenic medium in hypoxic condition. Collagen type II expression in scaffold without additional growth factor showed no statistically significant difference compared with the combination group in hypoxic condition. Cartilage tissue engineering has proven its effectiveness for cartilage regeneration. Decellularized biomaterial scaffold limited the use of growth factor resulting in better cost and resource efficiency.

1. Introduction

Cartilage of the knee joint has distinct characteristics compared to other tissues. Unlike the other tissues, cartilage of the knee joint has no vascularization (avascular), neural network (aneural), and lymphatic system (alymphatic) [1]. Any damage to cartilage tissue present complexity to regeneration process since no hematoma formation is expected to initiate the healing process intra-articular and intra-synovial [2].

Retrospective study had been done, and 60% cartilage injury had been found in routine diagnostic arthroscopy accidentally [3-6]. Another research on 931 athletes was diagnosed of a full-thickness focal chondral defect for 36% athletes [7]. Most of the cartilage injury occurred in young adult age, long-term study showed in 10 years observation 80% of it suffered from osteoarthritis [8].

Various methods of cartilage restoration have been well-known, including microfracture, osteochondral autograft transplantation, osteochondral allograft transplantation, and autologous chondrocyte implantation. Although autograft remains the gold standard method for repairing musculoskeletal tissue damage, this method causes donor site morbidity and has limited availability. Allograft and xenograft are potential sources of tissue, although they have some degree of risks in

enhancing immune responses and rejections associated with the presence of cellular components and the expression of major histocompatibility complexes (MHC) [9].

Autologous Chondrocyte Implantation (ACI) is a method for regenerating the articular cartilage to treat the cartilage defect problem. Although ACI had been established for years and showed promising results, this procedure still has weakness. One study mentioned cultured chondrocyte cells were eliminated before starting to produce any extracellular matrix [10]. Two steps of surgery were required to perform Autologous Chondrocyte Implantation procedure.

With its ability to regeneration and differentiation, stem cells provide new hope in the treatment of cartilage defects. Minimal invasive surgery could be performed in this procedure. Nevertheless, after further examination stem cells did not provide optimal results in treatment of cartilage defects. With the concept of tissue engineering in the last decade, attention and research to the field of stem cells is progressing very rapidly. However, in vitro research and the use of mesenchymal stem cells which have been widely practiced in the orthopedic field using conventional culture with normal oxygen tension (normoxia condition), which resulted in shock state and reduced viability of stem cells prior to transplantation [11].With the newer technique of scaffold decellularization, cellular components that can induce an immune response can be eliminated from allograft and xenograft scaffold, as well as being able to prove its potential as an alternative source of microenvironment stem cells [9]. However, the effectiveness of this technique in cartilage tissue engineering is still needed to be proved.

2. Research Methods

2.1 Tools and Materials

Equipment needed for this research are: 1 set of hypoxic chamber, Biological Safety Cabinet (BSC) class III, centrifuge equipped with brake button; incubator with humidity 5% CO_2 and temperature of 37°C; inverted phase contrast microscope with super long working distance condenser (SLWDC); 37°C waterbath, SCD sterill tubing welder; hand-held tube sealer, electric or manual pipette filter (0,1-10 mL); single channel pipette 10 µL - 1000 µL; Eppendorf Research series; pipette tips sterile aerosol separator; 10, 20, 200 and 1000 µL; hemocytometer with side cover: 15, 50 and 200 mL, conical sterile disposable centrifuge tube centrifuge; centrifuged disposable palpable centrifuge tubes: 1.5 mL, 1 L bottle Nelgen, 1 L sterile or sterile container to collect the harvest of the cell; serological sterile pipette 1, 2, 5 and 10 mL; vacuumtainer tube with sodium heparin; 175 cm^2 tissue culture; Pasteur plastic sterile pipette transfer; sterile pipette for aspiration, filter unit 0.22 µm pore size (milipore) 250 mL sterile; filter unit 0.22 pore size (Milipore) 500mL sterile; filter unit 0.22 µm pore size (Milipore) 1000 mL sterile; 10-tray cell plant, 6320 cm^2 of culture area.

The research materials used are Reagent for isolation: α-MEM with 1-glutamine, without ribonucleside or deoxyribinucleside (Sigma, Cat M0894); Fetal Bovine Serum (FBS) (Biowest, Cat S1650); 1 Glutamine, 200 mM (Initrogen); Penicillin G (10,000 units / mL) and streptomycin sulfate (10,000 µg / mL) in 0.85% NaCl (Sigma, Cat.P0781) solution; Ficol-Paque (Amersam Bisciences catalog # 17-440-02) similar (Sigma); Phospate buffered saline (PBS), without Ca^{2+} or Mg^{2+}, pH 7.4 (Merc); Tripsin (0.25%) - EDTA 4 NA (0.38 g / dL) (Merc). Solution used for isolation: Complete Culture Medium (CCM): 500 mL α-MEM; 100 mL FBS (Final consentration ~ 16.5%: 6 mL 1-glutamine (final concentration 2 mM) and 6 mL penicillin G and Streptomycyn Sulfate (final concentration 100 units / mL penicillin and 100 µg / mL streptomycin-optional); Filter up to 0.22 µm sterile unit filter: divided into aliquots and stored at 40°C for 2 weeks before the treatment is heated at 37°C .

Reagents for culture: low-glucose α-MEM (sigma, Cat M0894); 50 ml FCS selected for MSCs (Gibco / invitrogen); or alternative MSC Stimulatory Supplement (Stem Cell Technologies) or Bullet Kit for MSCs; 5 mL Antibiotic / Antimycotic mixture (Sigma, Cat A2942); Mix Solution, sterile filter and stored 40 C in dark room for 2 weeks new use. Additional Solution: Trypsin / EDTA: 0.05% trypsin / 0.23 mM EDTA (Merck); Phospate Buffer Saline (PBS) (Merck). MSCs

tissue culture plastic can be grown on a variety of tissue culture container. This research used products from NUNC and Corning.

2.2 Methods

This research was an experimental in vitro laboratory study of Bone Marrow Mesenchymal Stem Cells (BMSCs) culture on decellularized bovine cartilage scaffold under hypoxic condition. The experimental unit was divided into three different treatment groups, each group was divided by the treatment of normoxia and hypoxic conditions then evaluated at the same incubation period. The experimental unit in this study was mesenchymal stem cells taken from the bone marrow of male New Zealand white rabbit femoral bone, weighing at least 3 kg, aged between 6-9 months.

Implementation of this research was divided into 4 stages of research. First stage was the isolation of MSCs from the bone marrow of healthy male New Zealand rabbit strain. In second stage, MSCs were cultured on three different mediums of decellularized bovine cartilage scaffold, chondrogenic medium, and the combination of scaffold and chondrogenic medium that already contained growth plate. In third stage, stem cells culture were assigned into two different pressure of O_2 which were normoxia and hypoxic (21% and 1%) respectively. In final stage, stem cells differentiation were analysed into chondrocytes, which includes: (1) Collagen type II production on extracellular matrix formed (immunocytochemically and immunohistochemically); (2) Increased mature cholesterol (based on immunocytochemical and immunohistochemical SOX9 expression); and (3) Decreased formation of hypertrophic chondrocytes (based on immunocytochemical and immunohistochemical RUNX2 expression).

Sry-related high-mobility group box transcription factor 9 is abbreviated to SOX9, which is a marker for chondrogenic differentiation. Mature chondrocyte differentiation is marked by SOX9 expression in which higher expression leads to better result. Runt-related transcription factor 2 is abbreviated to RUNX2 which is a marker for osteogenic differentiation. In this research, hypertrophic chondrocyte differentiation is marked by RUNX2 expression in which lower expression leads to better result. Main product of the hyaline cartilage matrix is marked by collagen type II expression in which higher expression leads to better result [12].

3. Results and Discussion

Expressions of type II collagen, SOX9, and RUNX2 were compared different groups of stem cell differentiation in decellularized cartilage scaffolds without growth factor and combination of decellularized cartilage scaffold and chondrogenic medium under hypoxic and normoxia condition. Moreover, this research also conducted study using a monolayer culture plate for measuring the number of type II collagen, SOX9 and RUNX2 in the chondrogenic mediums exposed under hypoxic and normoxia conditions. Immunocytochemistry staining was performed to detect the expression of type II collagen and as well immunofluorescence examination was performed to detect the number of cells expressing SOX9 and RUNX2.

With immunocytochemistry staining, collagen type II expression was clearly seen with surrounding cells as shown in Figure 1. Then the expression of type II collagen was calculated on five planes with a 100x magnification light microscope.

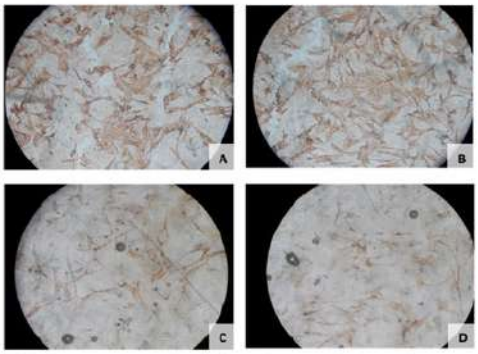

Figure 1. Expression of Collagen Type II Chondrogenic Medium with 100x Magnification. A-B. Within hypoxic condition, C-D. Within normoxia condition

As shown in Figure 1 and Figure 2, the amount of type II collagen expressed on chondrogenic medium in hypoxic conditions under constant surveillance showed much greater value than those in normoxia condition, and this difference was statistically significant ($p < 0.05$).

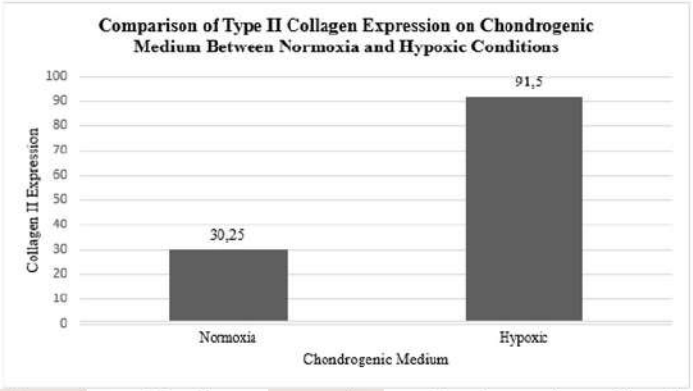

Figure 2. Comparison of type II collagen expression on chondrogenic medium between normoxia and hypoxic conditions

Furthermore, from Figure 3 and Figure 4, it is known that the number of SOX9 expressed and fluorescents by the chondrogenic medium in hypoxic conditions is more than those exposed to normoxia condition and this difference again was statistically significant ($p < 0.05$).

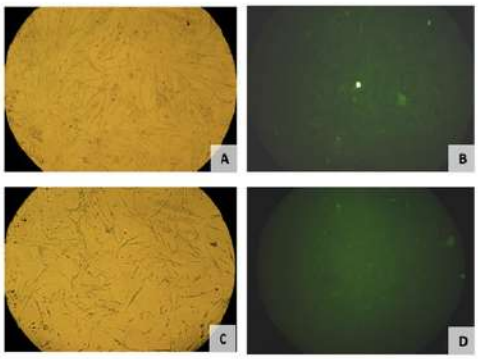

Figure 3. SOX9 Expression on chondrogenic medium. A-B. Within hypoxic condition, C-D. Within normoxia condition

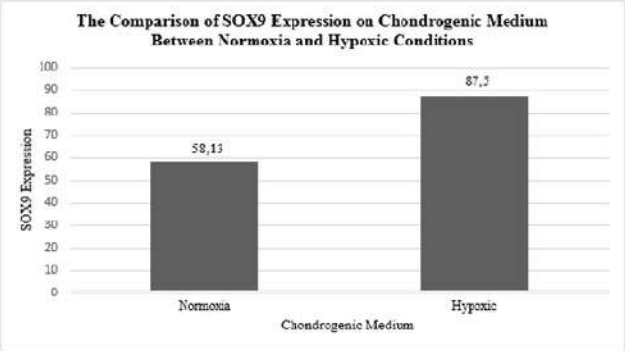

Figure 4. Comparison of SOX9 expression on chondrogenic medium between normoxia and hypoxic conditions

Different results were shown in Figure 5 and Figure 6 below whereby RUNX2 counts were expressed more in the chondrogenic medium under normoxia condition than fluorescent cells found in preparations exposed to hypoxic conditions. The result was statistically significant ($p < 0.05$).

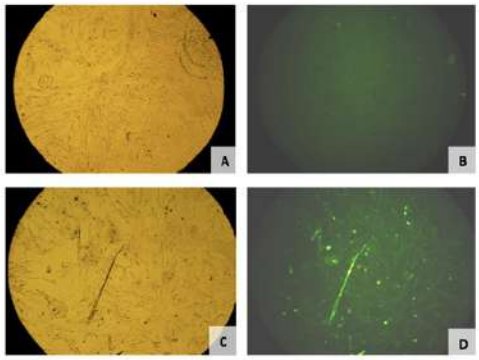

Figure 5. RUNX2 Expression on Chondrogenic Medium. A-B. Within hypoxic condition, C-D. Within normoxia condition

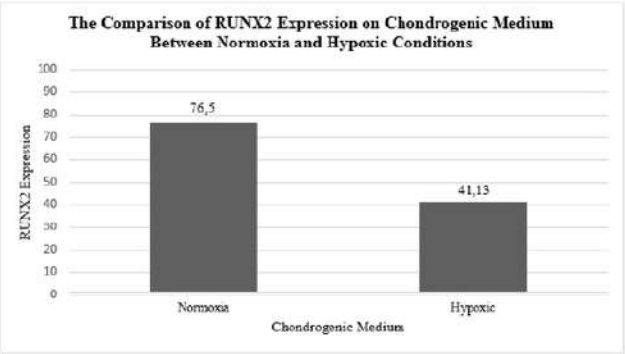

Figure 6. Comparison of RUNX2 expression on chondrogenic medium between normoxia and hypoxic conditions

From overall comparison, Figure 7 below showed that the largest expression of type II collagen was shown by the scaffold combined with chondrogenic medium in hypoxic conditions, but statistically if we compare to the scaffold only group in hypoxic condition, the result is not significant difference ($p > 0.05$).

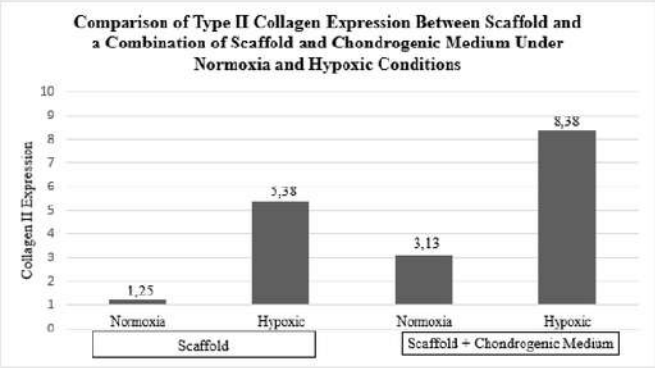

Figure 7. Comparison of type II collagen expression between Scaffold and a combination of scaffold and chondrogenic medium under normoxia and hypoxic conditions

The interesting point was shown in Figure 8 and Figure 9 which scaffolds combined with chondrogenic medium exposed to both hypoxic and normoxia conditions showed significant differences in SOX9 expression when compared with the scaffold only group ($p < 0.05$). But the expression of RUNX2 is inconsistently higher in scaffold only group within normoxia condition and higher in combination group within hypoxic condition.

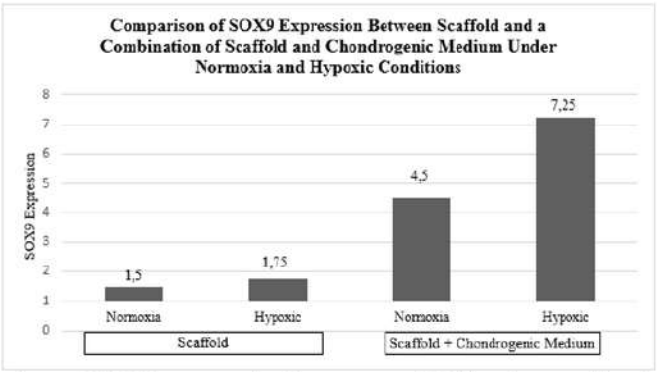

Figure 8. Comparison of SOX9 expression between scaffold and a combination of scaffold and chondrogenic medium under normoxia and hypoxic conditions

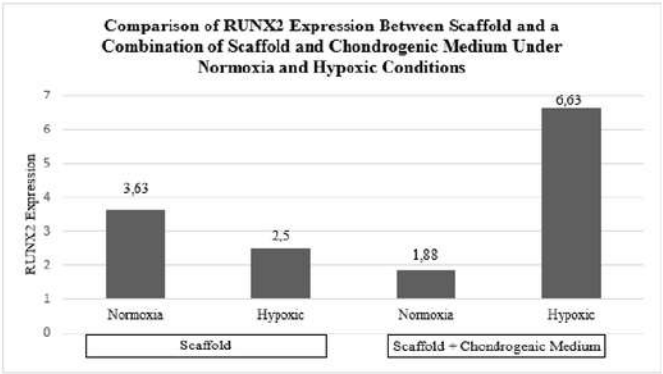

Figure 9. Comparison of RUNX2 expression between scaffold and a combination of Scaffold and chondrogenic medium under normoxia and hypoxic conditions

From a series of immunohistochemistry assay results of decellularized cartilage scaffold above, it was found that scaffolds in hypoxic conditions would express a higher amount of collagen, with SOX9 and RUNX2 statistically being no significant different from the scaffold in normoxia conditions. It was also found that in the decellularized cartilage scaffold combined with the chondrogenic medium group in hypoxic conditions expressed the higher number of collagen type II, SOX9, and RUNX2 levels compared to the combination of scaffold and chondrogenic medium exposed to normoxia conditions.

Stem cells underwent the differentiation phase in hypoxic condition showed better chondrogenic potency than in normoxia condition. This was supported by the fact that more SOX9 and collagen type II were expressed in stem cells cultured under hypoxic conditions. The hypothesis that hypoxic conditions inhibit the chondrocytes from becoming hypertrophic was demonstrated by lower RUNX2 expression in hypoxic conditions compared with normoxia ones.

Mesenchymal stem cells physiologically require optimal precondition in the form of low oxygen tension of 1-3% in bone marrow [13]. Low Oxygen tension (hypoxic condition) improves the differentiation process of stem cells into chondrocytes by inhibits terminal differentiation of stem cells into hypertrophic chondrocytes [14]. The terminal differentiation of chondrocytes into a hypertrophic state is correlated with the pathophysiology of osteoarthritis [15]. Therefore, keeping the chondrocytes phenotype within normal and preventing them from becoming into hypertrophic ones are essential in the treatment of cartilage defects.

The biomaterials used in this study were tested in previous studies under an electron microscope [16]. The decellularization technique was done physically, chemically, and enzymatically. With decellularization technique, it is expected that the biomaterial used in this research can be classified as raw material, which did not serve only as a scaffold but also contain a natural growth factors.

Decellularized cartilage bovine scaffold as a raw material also contains the growth factor were proven by the emergence of SOX9 and RUNX2 expressions in the study group of the scaffold only by seeding stem cells (Figure 10) without additional of growth factor from the outside. This proved the theory that there is an interaction between cells and biomaterials; the more physiological the biomaterials inhabited by stem cells, the better the growth and development of those cells [16, 17]. Stem cell differentiation into chondrocytes in decellularized cartilage bovine without additional growth factor was able to produce collagen type II, which is essential in the formation of hyaline cartilage.

Figure 10. Decellularized cartilage bovine after stem cell seeding was observed under an electron microscope

In the scaffold only group without additional growth factor, we found that the collagen type II was produced higher under hypoxic conditions. This supports previous research groups on chondrogenic mediums that hypoxic conditions stimulated chondrogenesis better than the normoxia ones. However, the exact mechanism in which the hypoxic conditions in the scaffold yield more type II collagen is still inexplicable. This is because SOX9 and RUNX2 expressions were not significantly different between hypoxic and normoxia conditions. The absence of growth factor in this sample group may also cause SOX9 and RUNX2 expressions did not fit the theory.

Group with a combination of scaffold and chondrogenic medium under hypoxic conditions have significantly higher type II collagen production than the others. This condition represents ideal

conditions that can be achieved in tissue engineering: optimally processed cells (hypoxic), good biomaterial scaffold (physiological scaffold), and availability of growth factor. The results of this study support the theory of tissue engineering triad (cells, scaffold, and growth factors) with the most superior results obtained in the combination group, both with the expression parameters of SOX9 and type II collagen. However, fulfilling all ideal requirements of the tissue engineering triad could spend many cost and resources despite its effectiveness.

The inconsistent phenomenon of RUNX2 expression on the scaffold in this study is interesting. In the scaffold group without additional growth factor in hypoxic conditions, RUNX2 expression decreased but was not statistically significant when compared to the normoxia condition in the same group. Meanwhile, in the combination group under hypoxic conditions, RUNX2 expression increased significantly compared to the normoxia condition in the same group. Meretoja et al stated that the medium change of the two-dimensional monolayer chondrogenic medium on the culture plate into a three-dimensional scaffold lead to oxygen gradient change [18]. Change in the oxygen gradient within the scaffold porosity causes some changes in the mechanism of hypoxic condition on differentiation of chondrocytes.

A study from Foldager et al compared the culture process on a two-dimensional monolayer medium with a three-dimensional medium under hypoxic condition. The study stated that there was a combination of positive effects between the three-dimensional culture mediums with hypoxic condition. This confirmed the theory that hypoxic condition is not the sole factor that affects the differentiation of chondrocytes in the three-dimensional medium [19]. Changing the culture medium from two-dimensional to three-dimensional itself is an important factor in inducing chondrogenesis [20]. This could explain the inconsistency of RUNX2 expression in this study and other studies [18-20].

Although it cannot be compared between groups of chondrogenic mediums with scaffold, researchers still can draw the conclusion that hypoxic conditions produced better quality and quantity than the normoxia ones because the expression of SOX9 and type II collagen was consistently produced higher in hypoxic conditions within all groups. In a semi-quantitative comparison between the scaffold groups and the combination group, the best results were obtained from the combination group.

If we analyze further the post hoc statistic result showed no significant difference in the production of type II collagen under hypoxic conditions between the scaffold group and the combination group. This is an important finding because the scaffold group under hypoxic conditions without additional growth factor meets the effectiveness and efficiency of tissue engineering. The author suggests for further study of this group with in vivo experiments because of its potential for reaching effectiveness and efficiency by limiting the use of growth factor.

4. Conclusion

Decellularized cartilage bovine matrix is a raw material that acts as a scaffold and contains the growth factor by providing good microenvironment to induce stem cell differentiation into chondrocytes. Hypoxic conditions increase the production of type II collagen by inhibiting terminal differentiation of stem cells to hypertrophy chondrocyte depicted by the increased amount of SOX9 expression and decreased expression of RUNX2. Mechanism how hypoxic condition affecting RUNX2 expression in three-dimensional tissue still cannot be explained.

Acknowledgements

The author would like to thank Tissue Bank and Regenerative Medicine Center Dr Soetomo Hospital Surabaya for biomaterial producing and Institute of Tropical Disease Airlangga University for stem cells processing.

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